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Leaf Surface Fungi of Early Blight [*Alternaria solani* (Ellis and Martin)] Infected and Non-Infected Leaves of Tomato [*Solanum lycopersicum* (L.)]

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A B S T R A C T

The tomato leaves infected with early blight recorded the highest leaf surface fungal species than non-infected leaves. In total twenty six fungal species (thirteen genera) were isolated from both diseased infected and non-infected leaf surface of tomato from seven districts of Meghalaya, following leaf impression, leaf washing and dilution plating and leaf washing and serial dilution plating methods. Among all the three methods used leaf impression method recovered the highest fungal population followed by serial dilution plating and leaf washing and dilution plating method. The predominant fungal species found both in infected and non-infected leaves were *Fusarium* sp., *Phoma* sp., *Penicillium* sp., *Aspergillus* sp. *Trichoderma* sp. and *Chaetomium* sp. Whereas, *Camarosporum* sp., *F. pallidoroseum* and *P. glabrum* were recovered only from healthy leaf samples. The further investigation can be done to find the antagonistic potential of leaf surface fungi against the major foliar diseases of tomato.

Introduction

Tomato (*S. lycopersicum* L.) is one of the most popular vegetable crops worldwide which share a great position in India as fresh vegetable. It belongs to the Solanaceae family which originated in the Andean region of South America. It is grown in a wide range of climate and the largest production centres are in southern and central part *i.e.*, Andhra

Pradesh, Madhya Pradesh, Karnataka, Gujarat, Odissa, West Bengal, Talengana, Chhattisgarh, Maharashtra and Bihar (Horticulture Statistics Division, Department of Agriculture, Cooperation and Farmers' Welfare, 2018). Area under tomato cultivation in the country is about 7.3 % of the total cropped land under vegetables and the area and production during 2017-2018 was 786 hectare (ha) and 19,377 Metric Ton (MT), respectively (Department of Agriculture Cooperation and Farmers Welfare, 2017). The agro-climatic condition of Meghalaya is also favourable for the cultivation of vegetables throughout the year. Among the vegetables, tomato is one of the most popular and widely grown vegetables crop in Meghalaya. In Meghalaya, it is cultivated throughout the year during rainy, winter and summer seasons. It occupies an area of 55.081 thousand ha with production of 35.51 MT during 2017-18 (Horticulture Statistics Division, Department of Agriculture, Cooperation and Farmers' Welfare, 2018).

Leaf surface is a habitat for a variety of microflora including pathogens and saprophytes. Leaf surface fungi are the mycota which grow on the leaf surfaces (Langvad, 1980). These group of fungi are categorized into two groups *i.e.*, casuals and residents (Norse, 1972). Casuals land on the surface of leaves but cannot grow whereas residents can multiply on the healthy leaf surface without noticeably affecting the host (Leben, 1965). Leaf surface fungi have not been fully studied and are still misused especially compared to rhizobacteria, root and seed endophytes and pathogenic microbes. Most of the work on leaf surface fungi was concerned with the pathogens or non-parasitic fungi of economically important trees (Dickinson, 1967; Pugh and Williams, 1968; Lamb and Brown, 1970; Pugh and Mulder, 1971; Bainbridge and Dickinson, 1972; Norse, 1972; Mishra and Dickinson, 1981; Cabral, 1985). This group of fungi were studied from mangroves (Kuthubutheen, 1981, 1984; Sivakumar and Kathiresan, 1990).

Due to the deposits of nutrients on the leaf surface is a favourable environment for millions of microbes. These microbial communities can be affected by internal and external agents like temperature, humidity, nutrient availability, leaf type and age and the presence of inhibitors (chemical compounds produced by the plant) (Andrews, 1991; Kinkel, 1997; De Jager *et al.,* 2001; Santamaria and Bayman, 2005; Evueh and Ogbebor, 2008). These microbes can either be beneficial or harmful to the host plant. Beneficial microbes cannot cause any disease symptoms unlike phytopathogens (Malfanova *et al.,* 2013). Therefore, leaf surface microflora is essential to study the environmental microbial diversity. It will also suggest the role of such community to the health and wellbeing of the plant as well as on members of the food chain that consume them. The three methods used in this investigation include leaf washing and dilution plating, leaf washing and serial dilution plating and leaf impression for culturing culturable fungi. The present investigation was carried out from one of the important vegetable crops *i.e.,* tomato. Three methods are employed to study leaf surface fungi (Lindsey, 1976).

Materials and Methods

Sample collection

Both early blight diseases infected and non-infected leaf samples of tomato were collected from 24 locations from seven districts *i.e.,* Ri-Bhoi, East Khasi Hills, West Jaintia Hills, East Jaintia Hills, North Garo Hills, West Garo Hills and South West Garo Hills of Meghalaya during the tomato

growing season from 2017-2018. Randomly twenty samples (twenty leaves per sample) from each field were collected in sterile poly-bags and taken back to laboratory for isolation of leaf surface fungi. Both the healthy and disease infected leaves were handpicked by holding the petiole only and placed into a separate fresh polyethylene bag, respectively. On bringing to the laboratory, department of plant pathology, SCP, CPGS, CAU, Meghalaya samples were immediately transferred to refrigerator (4±1°C) until further processing which normally was within 24 hours (h) of collection.

Isolation method

Three isolation methods were evaluated to characterize and identify the leaf surface fungi of tomato. The leaf surface fungi were isolated by leaf impressions (Dickinson *et al.*, 1974) and modified leaf washing method (Dickinson, 1971).

Leaf Impression method of isolation

Randomly, ten diseased infected and ten non-infected leaves were collected from the various locations. Collected samples were then rinsed for 10-15 times with tap water followed by sterilized distilled water (SDW) under a laminar air flow hood to remove externally loosely attached dusts and microbes.

The leaf impression was made by pressing both the leaf surfaces (upper and lower, separately) against potato dextrose agar (PDA) (Hi-media Ltd., Mumbai) in petri dishes to produce the leaf-imprints.

After leaving for an hour, leaves were then discarded and the plates were incubated at 28±1°C till colonies grow on the agar surface. The sample from every location was repeated for three times.

Leaf washing method of isolation

A modified leaf washing method was adopted (Dickinson, 1971) to estimate the leaf surface fungi. To estimate the leaf surface fungi, discs of 0.5 centimetres diameter was cut randomly from the washed tomato leaves with sterile cork borer aseptically. Twenty five discs for both the infected and non-infected leaves were placed separately in 250 ml conical flask containing fifty ml SDW. Then, the flask was shaken in a shaker for about 20 minutes to get a homogenous suspension of the microbial propagules. Sterilized Petri plates were poured with PDA medium and solidified. Using spread plate method one ml microbial suspension was pipetted out into the agar plates. Plates were then sealed with parafilm and incubated at 28±1°C for three days. Each sample was repeated for three times. Different colonies grown from leaf washes were subcultured, purified and fungi were preserved on PDA slants, respectively, at 4°C. The total microbial population per square cm of leaf surface of tomato was calculated by using the following formula (Vimala and Suriachandraselvan, 2006).

$$\text{Total number of microbes} = \frac{\text{Total number of microbes in 1 ml}}{\text{Total area of 25 discs x 2}} \times 100$$

(Area of 1disc=πr^2, where r is the radius of disc in cm)

Leaf washing and serial dilution plating technique

From the homogenous stock suspension of the microbial propagules as prepared by the above mentioned leaf washing method, a dilution series was made upto 10^{-5} of each sample suspension (healthy and infected stock suspension). One ml aliquots of the 10^{-3} dilutions were surface plated in triplicate on PDA media containing petri plates (de Jager *et al.*, 2001).

Identification and characterization of fungal isolates

Fungal colonies were subcultured after 3-4 days of incubation and pure cultures were transferred to PDA slants. Fungal isolates were studied based on cultural, morphological (Domsch *et al.,* 1980; Kharwar *et al.,* 2012) and microscopic characteristics *viz*. mycelium, conidiophore, spore structure etc. under microscope (Leica ICC50, Germany) by cover slip insertion method. And also those unidentified isolates were sent to National Centre of Fungal Taxonomy (NCFT), New Delhi for identification up to species level.

Results and Discussion

Isolation and identification

The diseased infected and non-infected leaf samples were collected from 24 locations from seven districts of Meghalaya. Altogether, twenty six fungal species of thirteen genera were isolated and identified on the basis of colony morphology, mycelia, sporangiophore and spore structure.

The predominant fungal species found both in infected and non-infected leaves were *Fusarium* sp., *Phoma* sp., *Penicillium* sp., *Aspergillus* sp. *Trichoderma* sp. and *Chaetomium* sp. Whereas, *Camarosporum* sp., *F. pallidoroseum* and *P. glabrum* were recovered only from the non-infected leaf samples (Table 1).

The genus *Phoma* recovered the total of six species followed by *Trichoderma* which is four species and *Fusarium* and *Penicillium* were three species.

The highest fungal isolates were recovered from the class Dothideomycetes *i.e.,* five genus and ten species. And least fungal isolates were recovered from the class Mucoromycotina and Oomycota *i.e.,* one genus and one species.

Among all the three methods used in this study, leaf impression infected upper leaves recovered the highest fungal population *i.e.*, 1.9 X 10^3 microbial population/cm^2 whereas the lowest fungal population was recovered from dilution plating healthy leaves *i.e.,* 6.2 X 10^2 microbial population/cm^2 (Table 2).

Though leaf impression method recovered higher fungal flora but it was difficult to assess the microbes quantitatively as very high density population was recovered. Also the microbial counting was difficult due to mixed nature of microbial population for their competition of nutrition and space. But, leaf impression method was a quick and simple method of isolation of leaf surface microflora (Dickinson, 1971).

Infected upper leaf surface recovered more fungal isolates than healthy leaf surface. Compared to all the three methods employed, leaf washing and dilution plating method was the most efficient method as it enabled the isolation of greater number and higher recovery of the same species. The morphological and cultural characteristics of all the fungal isolates were given in table 3.

Plant surfaces or internal tissues play as a home or niche for microorganisms. Therefore, plant surfaces are noted as a vital environment for microorganisms based on either a permanent (resident's epiphytes, endophytes or pathogens) and transient (unspecific epiphytic saprophytes) association (Forseca and Inacio, 2006).

In aerial leaf surface many organisms are found common in several crop plants. Only some species are restricted to a particular crop plant.

Table.1 Presence (+) or absence (–) of fungi isolated from diseased infected (I) and non-infected (NI) leaves of Tomato

Sr. No.	Name of the fungi	Isolation Methods							
		Leaf washing and dilution plating		Leaf washing and Serial dilution plating		Leaf Impression			
		I	NI	I	NI	I		NI	
						U	L	U	L
1	*Alternaria alternata*	+	–	–	–	+	–	–	–
2	*Botryodiplodia theobromae*	–	–	–	–	+	–	+	–
3	*Camarosporum* species novo.	–	–	–	–	–	–	–	+
4	*Cladosporium cladosporioides*	–	+	–	–	+	–	+	–
5	*Phoma glomerata*	–	+	+	–	–	+	+	+
6	*P. sorghina*	–	+	–	–	–	–	+	–
7	*P. lingam*	–	–	–	–	–	–	+	–
8	*P. exigua*	+	–	–	–	+	–	+	–
9	*P. herbarum*	–	–	+	–	+	+	–	–
10	*P. terrestris*	–	–	–	–	+	–	+	–
11	*Aspergillus niger*	+	–	+	–	–	+	–	+
12	*A. flavus*	+	+	–	–	–	–	+	–
13	*Penicillium* sp.	+	–	–	–	–	–	–	+
14	*Penicillium* sp.	+	–	+	–	–	–	–	+
15	*P. glabrum*	–	–	–	–	–	–	+	–
16	*Rhizopus* sp.	+	–	+	+	+	–	–	+
17	*Pythium aphanidermatum*	+	+	+	–	+	–	+	–
18	*Acremonium strictum*	–	–	–	–	+	+	+	+
19	*Chaetomium globosum*	–	+	–	+	–	–	+	+
20	*Fusarium pallidoroseum*	–	–	–	–	–	–	–	+
21	*F. oxysporum*	+	–	+	–	+	–	+	–
22	*F. solani*	+	–	–	–	–	+	+	–
23	*Trichoderma* sp.	–	–	–	–	–	–	+	–
24	*T. harzianum*	–	+	–	+	–	–	–	–
25	*T. viride*	–	+	+	+	–	–	–	–
26	*T. asperullum*	–	+	–	+	–	–	+	–

Table.2 Total microbial population per square cm of leaf surface of tomato [counts in colony forming units per millitre/leaf (cfu/ml)]

Sr. No.	Methods	Mean fungal population in 1 ml (cm^{-2})
1	Leaf impression-healthy upper leaves	1.1×10^3
2	Leaf impression-healthy lower leaves	9.9×10^2
3	Leaf impression-infected upper leaves	1.9×10^3
4	Leaf impression-infected lower leaves	1.3×10^3
5	Dilution plating-healthy leaves	6.2×10^2
6	Dilution plating-infected leaves	1.3×10^3
7	Serial Dilution plating-healthy leaves	7.6×10^2
8	Serial Dilution plating-infected leaves	1.7×10^3

Table.3 Morphological and cultural characteristics of leaf surface fungal isolates of tomato

Sr. No.	Fungal isolates	Class	Cultural characteristics					Microscopic observation		Nutrition
			Colour of isolate		Growth rate	Texture	Margin	Mycelium and Conidiophore	Conidia (Shape and colour)	
			Front	Reverse						
1	*A. alternata*	Dothideomycetes	Dark black	Dark black	S	P	Cr	Septate and branched brown colour hyphae. Pale brown straight conidiophores containing long chain conidia	Large, dark brown obclavate conidia in chain with short conical beak at the tip	Saprophyte
2	*B. theobromae*	do	Greyish black	Dark black	F	C	I	Conidiophores are hyaline, simple, sometimes septate, rarely branched cylindrical and arising from the inner layers of cells lining the pycnidial cavity. Pycnidia dark black colour	Immature conidia whitish with thin walls and mature conidia dark brown with septa and thick walls	do
3	*Camarosporum* species novo.	do	Greyish black	Greyish black	F	C	Cr	Septate and dark coloured mycelium. Conidiomata pycnidial	Conidia hyaline, aseptate and subcylindrical	do
4	*C.* cladosporioides	do	Dark grey	Dark black	M	V	Cr	Branched hyphae. Cylindrical branched conidiophore	Dark, single celled lemon shaped conidia	do
5	*P. glomerata*	do	Pale pink and greyish centre	Pale pink	F	WC	Cr	Dark brown septate hyphae. Chlamydospores in branched or un-branched chains. Chlamydospores showed both longitudinal and transverse septations as in commonly seen in the genus *Alternaria*.	Single celled hyaline, ovoid to ellipsoidal conidia. Conidia are bi-gulttulate (containing 2 oil drplets)	do
6	*P. sorghina*	do	White later greyish	Greyish	F	C	Cr	Hyphae septate and hyaline. Chlamydospores are unicellular, dark brown and botryoid-alternarioid shape. Pycnidia grey, globose and ostiolate	Conidia ellipsoidal to cylindrical, smooth, hyaline and aseptate	do
7	*P. lingam*	do	Whitish	Whitish	F	WC	Cr	Hyphae septate and hyaline. Pycnidia dark, globose and ostiolate	Conidia ellipsoidal, smooth, hyaline and single celled	do
8	*P. exigua*	do	Pale pink	Pale pink	F	C	Cr	No chlamydospores, dark walled pycnidia	Oblong to elliptic or often irregular hyaline conidia	do
9	*P. herbarum*	do	Whitish grey	Greyish	F	C	I	No chlamydospores, dark walled pycnidia, ostioles often with short beaks	Oblong to cylindrical with rounded ends hyaline conidia	do
10	*P. terrestris*	do	Pale pink	Reddish	F	C	Cr	Mycelium hyaline and septate. Pycnidia dark brown, subglobose, ostiolate and occur singly.	Conidia oblong to ovoid, hyaline and aseptate	do
11	*A. niger*	Eurotiomycetes	Dark black	Pale white	F	P	I	Mycelium septate, branched and hyaline. Erect, unbranched, single and club shaped conidiophore	Round shaped black coloured conidia, arranged in a long chain and single-celled	do
12	*A. flavus*	do	Greenish	Pale white	F	P	I	do	Round shaped green coloured conidia, arranged in a long chain and single celled	do
13	*Penicillium* sp.	do	Olive green with concentric circle	Creamish	S	FV	I	Hyphae septate, branched and hyaline. Erect, unbranched and septate conidiophore	One-celled hyaline and globose conidia	do
14	*Penicillium* sp.	do	Light blue	Creamish with reddish pigments	S	FV	I	do	do	do
15	*P. glabrum*	do	Brownish	Brownish	F	FV	I	do	do	do
16	*Rhizopus* sp.	Mucoromycotina	Greyish	Greyish	F	CC	Cr	Coenocytic with branched hyphae. Sporangia are supported by a large apophysate columella atop a long stalk, the sporangiophore. Sporangiophores arise from root-like rhizoids.	Sporangiospores globose, brown coloured and one-celled.	do
17	*P. aphanidermatum*	Oomycota	White	White	F	CF	Cr	Coenocytic and hyaline hyphae	Oogonia terminal, globose and smooth; antheridia intercalary, thick walled oospores and lobed sporangia	Facultative parasite
18	*A. strictum*	Sordariomycetes	White	Pale white	M	C	I	Hyphae are hyaline and produce mostly simple awl-shaped erect phialides with inconspicuous collarettes	Conidia hyaline, cynlindrical and single celled in chains or in conidial masses arised from short unbranched single phialides	Saprophyte
19	*C. globosum*	do	Olive green but later	Olive green	F	P	Cr	Mycelium often grows in conglomerate masses that resemble ropes. Ostiolar dark perithecia with	Flat lemon-shaped and olive brown ascospores within clavate ascomata	do

			stage become dark in colour					unbranched radiating hairs		
20	F. pallidoroseum	do	Yellowish	Yellowish	F	C	I	Mycelium septate and branched. Phialides are present	Macroconidia with 3 septa and microconida single-celled. Chlamydospores are present	do
21	*F. oxysporum*	do	White later become purplish	Purplish	M	C	I	Septate and branched mycelium. Conidiophores were elongated and sparsely branched	Macroconidia fusiform, slightly curved, pointed at tip and mostly three celled. Abundant microconidia, never in chains, mostly non-septate and ellipsoidal. Chlamydospores are also present	do
22	*F. solani*	do	White	Off white	M	C	I	Phialides are present. Chlamydospores are also common	Macroconidia are slightly curved, hyaline and broad. Microconidia cylindrical, hyaline and smooth and aseptate	do
23	*Trichoderma* sp.	do	Yellowish green	Creamish	F	P	Cr	Septate Branched with flask shaped phialides	Ellipsoidal, typically smooth with smooth walled conidia. Green coloured	do
24	*T. harzianum*	do	Green	Pale white	F	P	Cr	Flask shaped phialides and arranged in divergent groups of 2-4	Globose conidia	do
25	*T. viride*	do	Green	Pale white	F	P	Cr	do	do	do
26	*T. asperullum*	do	Green	Pale white	F	P	Cr	do	do	do

Note: S: slow, F: fast, M: medium, Cr: circular, I: irregular, P: powdery, C: cottony, V: velvety, FV: flat and velvety, WC: wooly colonies, CC: cotton candy; CF: cottony and fluffy

In this present investigation total twenty six fungal species from thirteen genera were recovered from three different isolation methods. It was found that the composition of tomato leaf surface fungi showed some similarities to other plant species like Egyptian wheat (Mazen *et al.,* 1985), *Spinacia oleracea* (Singh *et al.,* 1986), *Capsicum annuum* (Basha *et al.,* 2010), *Persea bombycina* (Bhuyan *et al.,* 2013) and *Abelmoschus esculentus* (Ogwu and Osawaru, 2014). The leaf impression method was the quickest and simplest method for the isolation of leaf surface microflora. But counting of colonies was also very difficult because of mixed nature of microbial population; may be slow growing colonies were hidden by fast growing microbes (Gunasekera, 1994).

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